Inhibition of Lipid Droplet Accumulation in Macrophages by Triterpenoids Produced from Torametes orientaris

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Abstract

Four triterpenoids 1—4 isolated from the fruit body of Torametes orientaris were found to inhibit lipid droplet accumulation in macrophages. From the biochemical analysis, compounds 2 and 3 inhibited selectively cholesteryl ester synthesis in macrophages, while compounds 1 and 4 showed inhibition of both cholesteryl ester and triacylglycerol synthases.

Key words lipid droplet accumulation; triterpenoid inhibitor; atherosclerosis; macrophage; neutral lipid synthesis; Torametes orientaris

In the early stage of atherosclerogenesis, macrophages penetrate into the intima, efficiently take up modified low density lipoprotein (LDL), store cholesterol and fatty acid as a respective form of cholesteryl ester (CE) and triacylglycerol (TG) in the cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall. Therefore, inhibitors of the macrophage foam cell formation would be expected to retard the progression of atherosclerosis.

During our course of screening for inhibitors of macrophage foam cell formation, new fungal metabolites of beauveriolides5—8) and phenochalasins A and B,9,10) and new actynomycetic metabolites of K97-0239A and B11) were discovered as inhibitors of lipid droplet formation in mouse macrophages. From further screening study, four known triterpenoids 1—4 (Fig. 1), reported to show weak antimicrobial or/and antitumor activity,12—14) were isolated from the fruit body of Torametes orientaris.

Here, we will describe the inhibitory activity of compounds 1—4 against lipid droplet accumulation in macrophages.

MATERIALS AND METHODS

Materials [1-14C]Oleic acid (1.85 GBq/mmol) was purchased from PerkinElmer Life & Analytical Sciences. Mature fruiting bodies of Trametes orientalis (YASUDA) IMAZ. were collected from Narusawa village in Yamanashi Prefecture of Japan.

Extraction and Isolation Fresh fruiting bodies of T. orientalis (200 g) were extracted with 85% EtOH (21, 5 times), and the solution was concentrated under reduced pressure and partitioned between EtOAc and H2O. The residue (10.8 g) obtained after removing EtOAc was fractionated by silica gel flash chromatography (80% CHCl3/acetone; 90%, 70% CHCl3/MeOH; MeOH) to obtain 7 fractions. Fraction 6 (1.60 g) was further separated by reverse-phase HPLC (95% MeOH/H2O), giving 1 (7.8 mg), 2 (3.5 mg), 3 (2.9 mg), and 4 (15.8 mg).

Assay for Lipid Droplet Accumulation in Mouse Macrophages Assay for lipid droplet formation in mouse peritoneal macrophages was carried out according to the method described previously.15) In brief, primary mouse peritoneal macrophages (2.5×105 cells in GIT medium) in each well of a 96-well plastic microplate (Corning Co.) were incubated in a humidified CO2 (5% v/v) atmosphere at 37 °C for 2 h. The medium was then replaced with 0.125 ml DMEM containing 8% (v/v) lipoprotein-deficient serum (LPDS), penicillin (100 units/ml) and streptomycin (100 mg/ml) (hereafter referred to as medium A). After another 2-h preincubation, 1.25 μl of a sample in methanol, and 5.0 μl of liposomes (phosphatidylcholine 1.0 μmol, phosphatidylserine 1.0 μmol, dicetylphosphate 0.20 μmol and cholesterol 1.5 μmol suspended in 1.0 ml of 0.3 M glucose) were added to each well. After a 14-h incubation, the cells were washed with PBS and then fixed by soaking in 10% formalin. Nuclei and intracellular neutral lipid droplets were then stained with hematoxylin and oil red O, respectively. The lipid droplet formation and morphological changes in macrophages were examined by a light microscopy (Vanox-S model, Olympus).

Assay for Neutral Lipid Synthesis in Mouse Macrophages Assay for [14C]CE, [14C]TG and [14C]PL syntheses from [14C]oleic acid in mouse macrophages was carried out according to the method described previously.15) In brief, mouse peritoneal macrophages (5×105 cells/0.25 ml medium A) were cultured in each well of a 48-well plastic microplate (Corning Co.), and then 2.5 μl of a test compound (MeOH solution) and 10 μl of liposomes together with 5 μl (1.85 kBq) of [14C]oleic acid (10% EtOH/PBS solution) were added to each culture. Following a 14-h incubation, the medium was removed, and the cells in each well were washed three times with PBS. The cells were lysed by adding 0.25 ml of PBS containing 0.1% (w/v) sodium dodecyl sulfate, and the cellular lipids were extracted by the method of
After concentrating the organic solvent, the total lipids were separated on a TLC plate (silica gel F254, 0.5 mm thick, Merck) and analysed with BAS2000 (Fuji Film).

### Other Biological Assays
Antimicrobial activity was tested using paper disks (i.d. 6 mm, ADVANTEC). Bacteria were grown on Mueller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after a 24-h incubation at 37 °C for bacteria and after a 48-h incubation at 27 °C for fungi and yeasts.

### RESULTS

#### Isolation of the Active Compounds
Isolation was guided by the result of bio-assay. Fresh fruiting bodies of *T. orientalis* were extracted with 85% EtOH. The extract, after concentrating the solvent, was partitioned between ethyl acetate and water. Since the EtOAc-soluble fraction showed inhibitory activity, this fraction was separated by repeated chromatography to obtain 1, 2, 3, and 4. All the spectral data of these compounds agreed with those reported previously.

#### Inhibition of Lipid Droplet Accumulation in Macrophages by Triterpenoids
In this macrophage assay, massive amounts of lipid droplets were accumulated in cytosols, which were observed microscopically after oil red O staining (control in Fig. 2A) when the macrophages were incubated with liposomes. Under the conditions, there were inhibition of lipid droplet accumulation in the presence of 1 (A), 2 (B), 3 (C) or 4 (D). After a 14-h incubation, cholesteryl [14C]oleate (1), [14C]triacylglycerol (2), and [14C]phospholipid (4) were separated on a TLC, determined with a bioimage analyzer as described in the "Materials and Methods" and plotted as % of control (without a drug).

#### Effect of Triterpenoids on CE and TG Syntheses in Macrophages
Lipid droplets formed in macrophages contain CE and TG. In the biochemical assay, the amounts of [14C]CE and [14C]TG synthetized from [14C]oleic acid by intact macrophages were measured (Fig. 3). Compounds 2 and 3 inhibited selectively [14C]CE synthesis with IC_{50} values of 32 and 40 μM, respectively, and they showed almost no effect on [14C]TG synthesis. However, 1 and 4 inhibited both [14C]CE and [14C]TG synthetises. The respective IC_{50} values of [14C]CE and [14C]TG synthetises were 20 and 42 μM for 1, and 10 and 13 μM for 4. Compounds 1, 2, 3, and 4 showed almost no effect on [14C]PL synthesis essential for cell growth even at 200 μM (Fig. 3).

#### Other Biological Activities
No antimicrobial activity of the triterpenoids was observed at a concentration of 10 μg/disk (2.0 mm) against the following microorganisms; *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces cerevisiae*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*.

None of these compounds showed cytotoxic, nematocidal and insecticidal activities at 0.2 mM (100 μg/ml).

### DISCUSSION
As described in this paper, four steroids 1—4 derived from triterpenes were found to inhibit lipid droplet accumulation in mouse macrophages. Several steroids and compounds with a steroid-like structure have been reported to inhibit lipid droplet accumulation in macrophages. For example, UI8666A (3β-[2-(diethylamine)ethoxy]-androst-5-en-17-one) and certain steroids inhibit cholesterol transport from lysosome to endoplasmic reticulum, and fungal epi-cochlinoquinone inhibits acyl-CoA: cholesterol acyltransferase (ACAT). Therefore, it might be plausible that compounds 1—4 inhibit the cellular cholesterol transport.
and/or ACAT activity in macrophages.

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